

# Nuclear protein transport is functionally conserved between yeast and higher eukaryotes

Philipp Wagner and Michael N. Hall

*Department of Biochemistry, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland*

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The ability of a yeast nuclear protein to be transported into the nucleus of a higher eukaryotic cell was investigated. Mcm1, a transcriptional activator protein from *Saccharomyces cerevisiae*, was microinjected into the cytoplasm of *Xenopus laevis* frog oocytes. Mcm1 was imported into the oocyte nucleus indicating that the machinery for nuclear transport is conserved from yeast to higher eukaryotes. Furthermore, by comparing the nuclear import of free proteins and protein–gold complexes, we found that protein–gold complex formation appears to partially and specifically inactivate the nuclear transport activity of Mcm1 and that nucleoplasmin is an exceptionally good nuclear import substrate.

Oocyte microinjection; Light microscope autoradiography; Electron microscopy; Mcm1; Nucleoplasmin; Protein–gold complexes

## 1. INTRODUCTION

Proteins destined for the nucleus are endowed with specific localization signals which enable them to be actively transported across the nuclear envelope (for reviews see [1,2]). Such nuclear localization signals (NLSs) have been identified by mutational analysis of nuclear proteins from both lower and higher eukaryotes; mutation of critical amino acid residues renders a nuclear protein cytoplasmic. In addition, when a protein, which normally is excluded from the nucleus, is tagged with an NLS either by gene fusion or by chemical crosslinking, the hybrid protein becomes localized to the nucleus.

NLSs mediate active transport into the nucleus through nuclear pore complexes [3–6]. Nuclear pore complexes are large, grommet-like structures (~ 120 nm in diameter, molecular mass ~ 124 MDa) [7] which traverse the outer and the inner membranes of the nuclear envelope forming a water-filled channel. The import of proteins into the nucleus consists of at least two steps. The first is a NLS-dependent binding of the nuclear import substrate to the cytoplasmic side of the nuclear pore complex followed by an ATP-dependent translocation through the pore [5,6,8,9].

NLSs have been identified in proteins from yeast to humans and there is no single consensus sequence (for reviews see [1,10]). However, there are some general descriptive rules. Nuclear localization signals (a) are

typically short sequences, usually not more than 8 to 10 amino acids, (b) contain a high proportion of positively charged amino acids (lysine and arginine) often associated with a proline, (c) can reside in any exposed region of a nuclear protein, (d) are not removed following localization and (e) can occur more than once in a given protein. The finding that many nuclear proteins contain two NLSs suggests that multiple signals could be a general feature of nuclear protein import. Although there is sequence similarity among NLSs from distantly related species, here we address the issue of whether there is functional conservation.

The finding that NLSs from higher eukaryotes also function in the yeast *Saccharomyces cerevisiae* [11–13] suggests that the mechanism for nuclear transport is conserved among all eukaryotes. However, this conclusion has been challenged by the inverse experiment in which a peptide containing a NLS of the yeast Mat $\alpha$ 2 protein was incapable of transporting a carrier protein into the nucleus of a higher eukaryote [14,15]. This suggested that the signal requirements in *S. cerevisiae* and higher eukaryotic cells may not be completely interchangeable.

To investigate whether nuclear protein import is indeed conserved between lower and higher eukaryotes, we injected a purified nuclear protein from yeast, the transcriptional regulatory protein Mcm1 from *S. cerevisiae* [16,17], into the cytoplasm of a higher eukaryotic cell and monitored its intracellular fate. The oocyte from the frog *Xenopus laevis* was chosen as a cell system for microinjection because it is well characterized for nuclear transport. The results indicate that the mechanism for nuclear transport is conserved between lower and higher eukaryotes.

*Correspondence address:* M.N. Hall, Department of Biochemistry, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Fax: (41) (61) 267 21 48.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of nuclear proteins

Mcm1 was obtained from S. Tan (ETH, Zürich). It was more than 90% pure and active, as determined by gel electrophoresis and DNA binding assays (S. Tan, personal communication). The active species of Mcm1 is a dimer (molecular mass 66 kDa) which forms stably in solution (S. Tan and T. Richmond, personal communication). Nucleoplasmin (NP) was purified in its native pentameric form (molecular mass 110 kDa) from *Xenopus laevis* frog oocytes according to Dingwall et al. [18]. *Xenopus laevis* frogs were a gift from L. Du Pasquier, Institute of Immunology, Basel. The proteins were radiolabeled by iodination with [<sup>125</sup>I]Na (100 mCi/ml; DuPont) using Iodobeads (Pierce) according to the manufacturer's recommendations. Typical specific activities were approximately 600 cpm/fmol for Mcm1 and 50 cpm/fmol for NP.

### 2.2. Preparation of protein-gold complexes

Gold particles with a diameter of 7 nm, prepared according to Slot and Geuze [19], were coated with Mcm1, NP or bovine serum albumin (BSA; molecular mass 66 kDa; Fraction V; Sigma) as follows. 50 µg of Mcm1, NP and BSA was first radiolabeled individually by iodination with ~ 0.1 mCi [<sup>125</sup>I]Na using Iodobeads in 150 µl 75 mM Tris-HCl, pH 7.5, for 20 min. A labeled protein solution was removed from the Iodobeads and mixed with 2 ml of colloidal gold (adjusted to a pH ~ 9 with 0.1 M K<sub>2</sub>CO<sub>3</sub>). After 1–2 min, 100 µl of 10 mg/ml BSA and 100 µl of 500 mM HEPES-KOH, pH 7.4, 50 mM MgSO<sub>4</sub>, 30 mM CaCl<sub>2</sub>, 250 mM potassium acetate were added to a mixture and the protein-gold complexes were separated from free <sup>125</sup>I and uncomplexed protein by gel filtration on a Biogel P-300 column (Bio-Rad) equilibrated with column buffer (1 mg/ml BSA in 50 mM HEPES-KOH, pH 7.4, 5 mM MgSO<sub>4</sub>, 3 mM CaCl<sub>2</sub>, 25 mM potassium acetate). The complexes were concentrated by spinning at 40,000 × g in a Sorvall SS-34 rotor for 30 min at 4°C and taken up in a small volume (~ 150 µl) of column buffer containing 0.01% sodium azide. To determine the relative coupling ratio of protein per gold complex, 5 µl of the protein-gold suspensions were examined by SDS-PAGE in parallel with a known amount of radioiodinated, but uncomplexed protein samples. Autoradiographs were scanned in a Molecular Dynamics 300A Computing Densitometer and the relative coupling ratio was calculated from the amount of radioactivity in the different samples. Approximately seven times more monomers of NP were bound per gold particle than monomers of Mcm1.

For observation of microinjected substrates by electron microscopy, nonradioactive protein-gold complexes were used. In this case, after complex formation, the protein-gold complexes were dialyzed overnight at 4°C against intracellular medium [3] consisting of 102 mM KCl, 11.1 mM NaCl, 7.2 mM K<sub>2</sub>HPO<sub>4</sub>, 4.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0, and centrifuged at 40,000 × g for 45 min at 4°C. The pellets were used directly for microinjection without further dilution.

### 2.3. Microinjection of *Xenopus* oocytes

*Xenopus* oocytes were microinjected with Mcm1, NP and BSA complexed to colloidal gold<sub>7nm</sub> for electron microscopy. The same set of proteins but uncomplexed and labeled with <sup>125</sup>I were microinjected and used for light microscope autoradiography.

Pieces of ovaries were surgically removed from female *Xenopus laevis* frogs. The ovaries were kept at 4°C in modified Barth's saline (88 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM NaHCO<sub>3</sub>, 15 mM HEPES-NaOH, pH 7.6) supplemented with streptomycin and penicillin until use. For injection, fully grown oocytes were dissected from the ovaries in modified Barth's saline at room temperature. Single oocytes were placed on an agarose slab and microinjected cytoplasmically with approximately 100–200 nl of substrate solution aimed toward the yolky vegetal pole. The orifices of the glass capillaries had a diameter of 15–30 µm. For each substrate to be tested, at least 15–20 oocytes were injected. Following injection, the oocytes were maintained at room temperature in modified Barth's saline for 5.5 h after which they were fixed in 2.5%

glutaraldehyde, 2% formaldehyde, 0.1 M cacodylate buffer, pH 7.4, 2% Me<sub>2</sub>SO, 1 mM CaCl<sub>2</sub> for 2 h at room temperature or overnight at 4°C and then processed further for embedding in Epon.

### 2.4. Embedding of *Xenopus* oocytes in Epon

Fixed oocytes were washed in 0.1 M cacodylate buffer, pH 7.4, dehydrated through a graded series of ethanol concentrations (30%, 50%, 70%, 90%, 2 × 100%; 1 h for each step on a rocking platform), followed by propyleneoxide for 30 min. Infiltration was with Epon/propyleneoxide, 1:1 for 1 h and 2:1 overnight with open vials on a rotating disc. The next day, fresh Epon was added and changed after 1 h before the oocytes were put in gelatin capsules. Polymerization of the resin was at 40°C for 24 h and at 60°C for 3 days.

Oocytes which were intended for electron microscopy were cut open at the vegetal pole and the follicular membrane was removed after aldehyde fixation to ensure good penetration with the resin. In addition, these oocytes were postfixed in 1% OsO<sub>4</sub>, 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C.

### 2.5. Light microscope autoradiography of *Xenopus* oocytes

Autoradiography was performed according to the techniques described by Rogers [20]. Semi-thin sections (1–2 µm) were cut on a microtome (Reichert) and placed on microscope glass slides cleaned with ethanol and coated with gelatin. Placed on every slide were several sections from oocytes injected with the different radioiodinated proteins, including sections from noninjected oocytes to control for background formation of silver grains. In the dark, the slides were dipped into Kodak NTB-2 nuclear-type emulsion and, after wiping the back of the slides with a paper tissue, put on an ice-cold metal plate for 15–20 min and then placed at room temperature for an hour. The slides were dried overnight at room temperature in open exposure boxes containing silica gel. The next day, the boxes were closed, wrapped in two black plastic bags and placed at 4°C for exposure. After 4 days at 4°C, the boxes were equilibrated to room temperature for 3 to 4 h and the slides were developed in Kodak D-19 (1 part diluted with 3 parts water) for 4 min, rinsed in water, fixed for 8 min in 30% sodium thiosulphate and washed in running tap water for 15 min. The slides were viewed under a Zeiss photomicroscope II.

### 2.6. Quantitation of nuclear transport in *Xenopus* oocytes

#### 2.6.1. Electron microscopy

Thin sections (50–80 nm) were cut on a microtome (Reichert), taken up on parlodion-carbon coated nickel grids, and contrasted with 2% aqueous uranyl acetate for 30 min and lead acetate [21] for 5 min. Sections were viewed in a Zeiss EM10 electron microscope. For every substrate, several randomly selected pictures were taken from thin sections of three injected oocytes. For each picture, gold particles within an equal area (adjacent to the nuclear envelope) over the nucleus and the cytoplasm were counted and summed separately. The nuclear to cytoplasmic ratio was calculated by dividing these two figures.

#### 2.6.2. Light microscopy

For every substrate, pictures were taken at high magnification from the nucleus and the cytoplasm of five injected and sectioned oocytes. Silver grains were counted on equal, randomly selected areas over the nucleus and the cytoplasm and summed separately. Although at low magnification (79 ×) silver grains were seen only over sections from oocytes injected with <sup>125</sup>I-labeled proteins, at higher magnification (500 ×) slightly smaller silver grains were also detected over noninjected oocytes representing background formation of silver grains. As these smaller grains could not be clearly distinguished from the other grains, they were included in the counting. To calculate the nuclear to cytoplasmic ratio, the number of nuclear grains was divided by the number of cytoplasmic grains after correcting for background formation in each compartment, i.e. the number of grains from background formation was subtracted from the total number of grains counted over each area. In the case of nucleoplasmin, there was such a high

density of silver grains over the nucleus that it was sometimes difficult to distinguish individual silver grains. Therefore, the total number of silver grains counted for nuclear  $^{125}\text{I}[\text{NP}]$  may slightly underestimate the actual amount.

### 3. RESULTS

#### 3.1. Microinjection of *Xenopus* oocytes with protein-gold complexes

To investigate nuclear transport of the yeast nuclear protein Mcm1 in frog oocytes, *Xenopus* oocytes were first injected with protein-gold complexes. BSA-gold was used as control for a nonnuclear protein and NP-gold as a positive control for nuclear import [3]. After complex formation, the protein-gold complexes were dialyzed against intracellular medium, concentrated by centrifugation and injected into fully grown oocytes. The injected oocytes were incubated at room temperature for 5.5 h, a time long enough to ensure equal dispersal of the injected material [18]. After incubation, the oocytes were fixed and processed for electron microscopy.

We found that during the time of incubation, BSA-gold was almost completely excluded from the nucleus (Table I) whereas NP-gold was imported very well (Fig. 1 and Table I). The result for Mcm1 was intermediate; Mcm1-gold complexes were clearly imported into the oocyte nucleus when compared to the nonnuclear substrate BSA-gold (Fig. 2 and Table I). In addition, the nuclear to cytoplasmic ratio of 0.056 for Mcm1-gold is similar to the ratio, 0.077, for the nuclear import of SV40 T-antigen-gold as observed in a comparable experiment by others [22]. However, the nuclear to cytoplasmic ratio for Mcm1-gold was much lower than for NP-gold (Table I), suggesting that NP-gold is an exceptionally good nuclear import substrate. NP-gold was also often seen accumulated in nuclear pore complexes whereas this was never observed for Mcm1-gold (Figs. 1 and 2). This difference may be due to the two substrates having different rate-limiting steps in the import reaction: NP-gold could be rate-limited in passage through the pore whereas the rate limiting step of

Table I

Intracellular distribution of protein-gold<sub>7 nm</sub> complexes microinjected into *Xenopus* oocytes

Substrate	Particles in nucleus (n)	Particles in cytoplasm (c)	n/c
BSA-gold <sub>7 nm</sub>	2	908	0.0022
Mcm1-gold <sub>7 nm</sub>	81	1,452	0.056
NP-gold <sub>7 nm</sub>	1,576	768	2.1

Frog oocytes were injected with protein-gold complexes and incubated for 5.5 h at room temperature after which they were processed for electron microscopy. For each substrate, gold particles were counted on equal areas over nucleus and cytoplasm of three injected oocytes.

Table II

Intracellular distribution of  $^{125}\text{I}$ -labeled proteins microinjected into *Xenopus* oocytes

Substrate	Silver grains in nucleus (n)	Silver grains in cytoplasm (c)	n/c
$^{125}\text{I}$ BSA	323	464	0.70
$^{125}\text{I}$ Mcm1	2,014	220	9.2
$^{125}\text{I}$ NP	5,858	149	39

Frog oocytes were injected with  $^{125}\text{I}$ -labeled proteins and incubated for 5.5 h at room temperature after which they were processed for light microscope autoradiography. For each substrate, silver grains were counted on equal areas over nucleus and cytoplasm of five injected oocytes.

Mcm1-gold import could be the initial binding to the pore.

One simple explanation for the relatively low level of transport of Mcm1-gold compared to NP-gold could be that the number of NLSs which were exposed per protein-gold complex was lower for Mcm1 than for NP. The efficiency of nuclear transport has been shown to increase with increasing number of NLSs per nuclear substrate [18,22–24]. Therefore, we determined the relative coupling ratio of protein per gold colloid for NP and Mcm1 by complexing radiolabeled proteins to the gold particles (see section 2). We found that there were indeed differences in the coupling of Mcm1 and NP to gold particles; approximately seven times more monomers of NP bound per gold particle than monomers of Mcm1 (not shown), corresponding to about three times more NP pentamers bound per gold particle than Mcm1 dimers. Because the coupling ratio of protein per gold colloid is an inherent property of every protein-gold complex and cannot be controlled without changing critical parameters like gold particle size [25], we injected free, uncomplexed proteins in order to measure nuclear import under more comparable conditions for the different substrates.

#### 3.2. Microinjection of *Xenopus* oocytes with radioiodinated proteins

To compare nuclear import under more comparable conditions, purified free proteins (BSA, NP and Mcm1) were radiolabeled with  $^{125}\text{I}$  and microinjected into *Xenopus* oocytes. Incubations were again for 5.5 h at room temperature, after which the oocytes were fixed and processed for light microscope autoradiography. With this method, a radiolabeled protein can be localized in a tissue section by the silver grains which are generated in an overlaid photographic emulsion. The silver grains can then be quantified in the light microscope at high magnification (see section 2).

The results showed, first, that  $^{125}\text{I}$ BSA dispersed throughout the cytoplasm and was mostly excluded from the nucleus, as seen by a nuclear to cytoplasmic

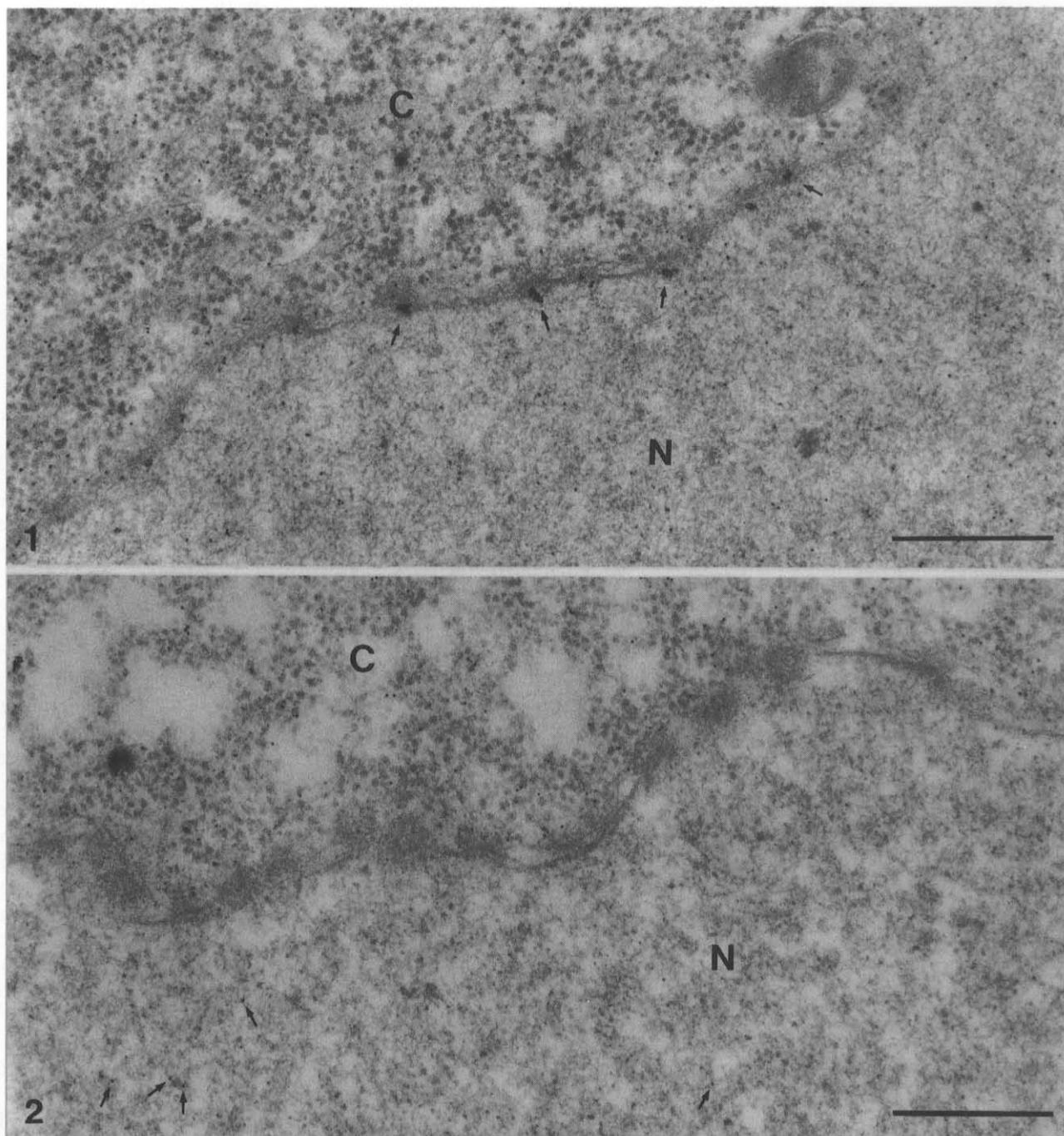
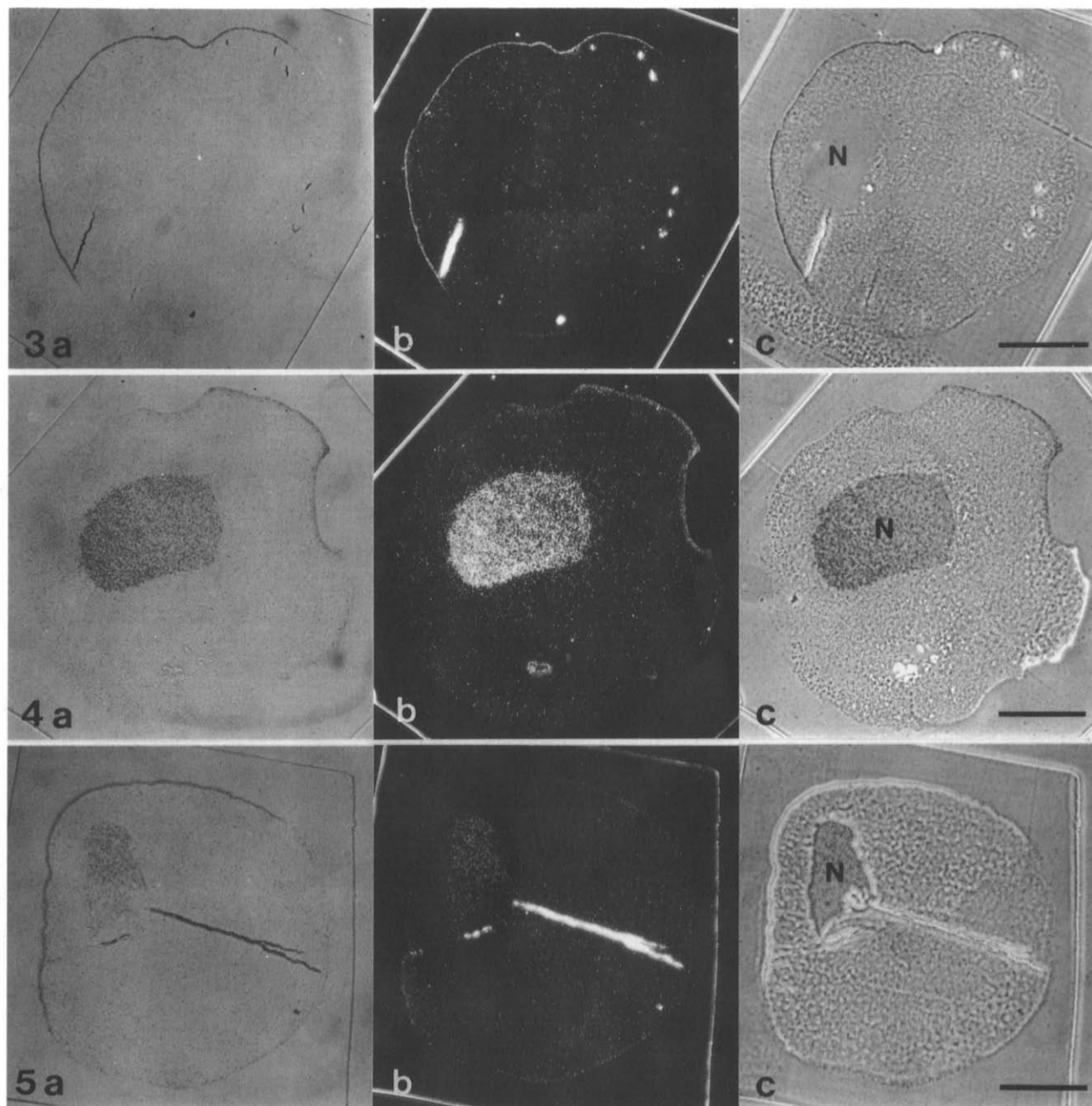


Fig. 1. Electronmicrograph of a section through a frog oocyte injected with NP-gold. NP-gold<sub>7nm</sub> complexes are transported across the nuclear envelope and accumulate inside the nucleus of *Xenopus* oocytes ( $t = 5.5$  h). Often several NP-gold complexes (arrows) are seen being transported through a single nuclear pore complex in the nuclear envelope. C, cytoplasm. N, nucleus. Bar, 500 nm.

Fig. 2. Electronmicrograph of a section through a frog oocyte injected with Mcm1-gold. Mcm1-gold<sub>7nm</sub> complexes are transported into the nucleus of *Xenopus* oocytes ( $t = 5.5$  h). Some of the nuclear Mcm1-gold complexes are indicated by arrows. C, cytoplasm. N, nucleus. Bar, 500 nm.

ratio of 0.7 (Fig. 3 and Table II). Second, [<sup>125</sup>I]NP was imported exceptionally well into the nucleus of the oocytes resulting in such a high density of silver grains over the nucleus (Fig. 4) that individual grains could sometimes not clearly be distinguished even at high

magnification (not shown). Therefore, the actual nuclear to cytoplasmic ratio for [<sup>125</sup>I]NP may have been slightly higher than the determined value of 39, but we estimate by only a factor of  $\leq 2$ . Third, in contrast to the Mcm1-gold complex, <sup>125</sup>I-labeled free Mcm1 was



Figs. 3–5. Light microscope autoradiography on sections from frog oocytes injected with  $^{125}\text{I}$ -labeled proteins. Autoradiographs of sections of *Xenopus* oocytes injected with  $^{125}\text{I}$ -labeled proteins and incubated for 5.5 h as seen in the light microscope at  $79\times$  magnification by different modes of illumination. (a) Brightfield photo. (b) Darkfield photo. (c) Phase-contrast photo. N, nucleus. Bar, 0.5 mm. 3(a–c): [ $^{125}\text{I}$ ]BSA. 4(a–c): [ $^{125}\text{I}$ ]NP. 5(a–c): [ $^{125}\text{I}$ ]Mcm1.

efficiently imported and concentrated in the nucleus of the oocytes (Fig. 5) resulting in a nuclear to cytoplasmic ratio of 9.2 (Table II). This ratio for Mcm1 is similar to the ratio, 6.5, observed for localization mediated by the SV40 T-antigen NLS [26].

#### 4. DISCUSSION

By microinjection experiments, we have shown for the first time that a NLS of a yeast protein can be

recognized by the nuclear transport machinery of a higher eukaryotic cell; Mcm1, a nuclear protein from *S. cerevisiae*, is transported into the nucleus of a *Xenopus laevis* frog oocyte. Previously, it had been shown that the NLS from SV40 T-antigen when fused to a nonnuclear protein could direct the passenger protein to the yeast nucleus in vivo [11,13] and in vitro [12]. Similarly, two NLSs of the VirD2 protein from *Agrobacterium tumefaciens*, which are similar to the NLSs of NP and SV40 T-antigen, target the VirD2 protein to the nucleus



of plant and yeast cells [27–29]. Taken together, these results demonstrate that the machinery for nuclear protein transport is functionally conserved among all eukaryotes. This is consistent with previous findings that NLSs from yeast to vertebrates and viruses of higher eukaryotic cells follow some general rules (see section 1) although they do not show a strict consensus sequence.

The nuclear accumulation of Mcm1 was most likely not due to passive entry and selective retention of dissociated Mcm1 monomers because the Mcm1 dimer is stable and because even smaller proteins with intranuclear binding sites have been shown to be unable to accumulate in the nucleus by diffusion [30,31].

Why is a peptide containing a NLS of the yeast protein Mat $\alpha$ 2 incapable of transporting a carrier protein into the nucleus of a mammalian cell (see section 1; [14,15])? The Mat $\alpha$ 2 protein contains two structurally distinct NLSs which have different efficiencies in nuclear import, and the signal tested in mammalian cells works only very weakly in yeast [9]. Thus, the lack of import observed in mammalian cells could be due to a normal variation in the import efficiency of a given signal in different cell types. Alternatively, the Mat $\alpha$ 2 signal could be a specialized yeast signal, or the tested peptide had an incorrect conformation.

Nucleoplasmin is an exceptionally good nuclear import substrate. We found that, for a given time of incubation, the frog protein NP always localized to the frog oocyte nucleus better than the yeast protein Mcm1 and, as observed by others, SV40 T-antigen. While one reason for the difference in transport activities could be a difference in the affinities of the respective NLSs for an import receptor, another possibility is the number of NLSs per import substrate. The extent of nuclear accumulation depends on the number of NLSs per nuclear protein [18,22–24]. NP is a pentameric protein whereas Mcm1 is a dimer. Assuming that each monomer contains one complete NLS, the difference in the state of oligomerization could account for the observed difference in nuclear uptake of the free, radiolabeled proteins (see Table II).

With regard to protein–gold complexes, the number of NLSs per substrate is primarily determined by the coupling ratio of protein per gold particle. The coupling ratio was approximately seven times higher for NP (monomer) compared to Mcm1 (monomer). However, the coupling ratio alone could not explain the observed large difference in transport activity of NP– versus Mcm1–gold (see Table I). The accessibility of the NLS on the gold particle could also be a factor. Thus, the nuclear transport activity of Mcm1 may have been partially and specifically inactivated after complex formation to colloidal gold, e.g. by masking one of the two NLSs of the Mcm1 dimer. Such steric inactivation of some NLSs may affect NP much less since it contains five NLS per native pentamer. Comparative studies

with different protein–gold complexes should therefore be interpreted with caution.

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